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Cellular internalization of lactoferrin in intestinal epithelial cells

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Abstract

We studied the cellular internalization of lactoferrin (Lf) in an intestinal epithelial cell line, Caco-2, to investigate the mechanism of biological actions of ingested Lf. RT-PCR and Western blotting analyses revealed that differentiated Caco-2 cells express LfR mRNA and its protein with a 34 kD molecular weight under reducing conditions. Biotin-labeled Lf showed specific binding to the cellular membrane of differentiated Caco-2 cells with a dissociation constant (K_d) of 0.16 μM. The cellular internalization of Lf was studied in differentiated Caco-2 cells grown as monolayers on Transwell inserts, and compared to that of human transferrin (Tf). After labeling with fluorescent dye, either Lf or Tf was added to Caco-2 cells from the apical side or the basolateral one. Laser scanning confocal microscopy showed that labeled Lf was internalized only from the apical side and localized to the nuclei. On the other hand, labeled Tf was internalized from the basolateral side, not from the apical side, and localized in the cytoplasm. The internalization of labeled Lf was inhibited by excess of unlabeled Lf, but not of Tf. The internalization of labeled Lf, but not of labeled Tf, was also suppressed by heparin. This indicates that a heparin-binding site in the N-terminal region of Lf could be important for the internalization of Lf. These findings suggest that ingested Lf might be internalized by the intestinal epithelium in a manner different from Tf and might function in the nucleus.

Introduction

Milk, especially human milk, contains an iron-binding protein, lactoferrin (Lf). Lf is known to be comparatively resistant against proteolysis by trypsin (Brock *et al.* 1976), and it has been shown that a significant proportion of Lf can survive digestion in infants (Davidson & Lönnerdal 1987). It has been reported that orally administered Lf has systemic effects on the immune system in humans and other animals (Brock 2002; Ward *et al.* 2002). Although the mechanisms behind the systemic effects have not been revealed, it seems logical to hypothesize that a specific receptor for Lf (LfR) expressed in small intestine is involved in the uptake of Lf. A LfR, a specific binding protein for Lf, has been identified in brush-border membranes of fetal and infant intestines (Kawakami & Lönnerdal 1991). The LfR has been shown to be involved in the cellular uptake of iron bound to Lf from milk and has also been suggested to be involved in other functions of Lf (Suzuki *et al.* 2001). LfRs have been identified and characterized in several other mammalian cell types or tissues such as monocytes (Birgens *et al.* 1983), lymphocytes (Mazurier *et al.* 1989), and liver (Retegui *et al.* 1984, McAbee & Esbensen 1991, Ziere *et al.* 1992). In mammalian cells, cellular internalization (Ziere *et al.* 1992, Bi *et al.* 1996, Sasaki *et al.* 2002) and nuclear localization of Lf (Garré *et al.* 1992) has been demonstrated, suggesting that Lf acts on DNA in the nucleus (He & Furmanski 1995, Son *et al.* 2002). We thus hypothesized that ingested Lf is internalized and exerts systemic functions in the intestinal epithelium, which are likely to be mediated by the LfR. Therefore, we examined the cellular internalization of Lf in an intestinal epithelial cell model to

study the mechanisms behind the systemic effects of ingested Lf.

Materials and methods

Cell culture

Human colon carcinoma Caco-2 cells (ATCC HTB-37) were cultured in MEM supplemented with 20% (v/v) fetal bovine serum, 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids and 1.0 mM sodium pyruvate and maintained at 37 °C in 5% CO_2.

RT-PCR

Total RNA was isolated from Caco-2 cells cultured for several different periods (3–26 days). First-strand cDNA was synthesized from total RNA by using reverse transcriptase (Invitrogen, Carlsbad, CA, USA). LfR cDNA was amplified by a specific primer pair for human LfR (5′-ACCCAAGGAAAGTGCAGCTGAGA-3′ and 5′-GTTCCCTCCCACAAAACTCTCAACGA-3′). The PCR products were electrophoresed in 1.5% agarose gels. The gels were stained with ethidium bromide and the pictures of them were taken by a CCD camera (Bio-Rad Laboratories, Hercules, CA, USA) under a UV lamp.

Western blotting

Cell lysates were prepared from Caco-2 cells cultured for several different periods by using commercial cell lysis buffer (Pierce Biotechnology, Rockford, IL, USA). Proteins in cell lysate were separated by SDS-PAGE and transferred to PVDF membranes (Bio-Rad Laboratories). The membranes were blocked with Superblock (Pierce Biotechnology) and incubated with a 1:50 dilution of rat anti-recombinant human lactoferrin receptor antiserum and then incubated with a 1:2,000,000 dilution of horseradish peroxidase-conjugated goat anti-rat IgG antibody (Antibodies Incorporated, Davis, CA, USA). West Femto substrate and X-ray films (Pierce Biotechnology) were used to detect the protein bands representing LfR.

Membrane binding assay

Recombinant human Lf (Agennix, Houston, TX, USA) was labeled with biotin by using a commercial biotinylation kit (Pierce Biotechnology). The cellular membrane was isolated from Caco-2 cells cultured for 19 days. The surface of the ELISA plates (Nalge Nunc International, Naperville, IL, USA) was coated with the membrane at 4 °C overnight and washed with washing buffer. Each well was blocked with gelatin at 37 °C for 1 h and washed with washing buffer. Biotin-labeled Lf was diluted with binding buffer to several concentrations and added to membrane-coated plates in the presence or absence of a 20-fold excess of unlabeled Lf. After incubation at 37 °C, each well was washed with washing buffer. The amount of membrane-bound labeled Lf was quantified by avidin-linked peroxidase (Vector Laboratories, Burlingame, CA, USA) and *o*-phenylenediamine dihydrochloride (OPD) substrate (Sigma, St. Louis, MO, USA).

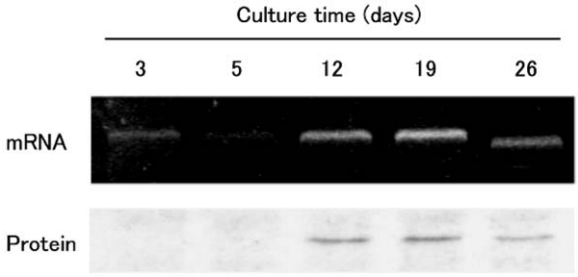

Fig. 1. Lactoferrin receptor (LfR) expression in Caco-2 cells cultured for different times. LfR mRNA expression was analyzed by RT-PCR. LfR protein expression was analyzed by Western blotting under reducing conditions. The bands were detected at a molecular weight of 34 kDa.

Cellular internalization studies of Lf

Lf and iron-saturated transferrin (Tf) (Sigma) were labeled with Alexa Fluor 488 fluorescent dye (Molecular Probes, Eugene, OR, USA). Caco-2 cells were grown as monolayers on Transwell inserts (Corning, Corning, NY, USA) for 19 days. Labeled Lf or Tf was diluted in PBS to 100 μg/ml and added to differentiated Caco-2 cells from either the apical or the basolateral side. After incubation for 30 min at 37 °C, cells were washed with cold PBS and fixed in 4% paraformaldehyde. The nucleus was stained with TO-PRO-3 fluorescent dye (Molecular Probes). The internalization of labeled Lf and Tf was also examined in the presence or absence of 10 mg/ml unlabeled Lf, 10 mg/ml unlabeled Tf or 1 mg/ml heparin (Sigma). The samples were then embedded in ProLong antifade reagent (Molecular Probes) on slide glasses. Images of the fluorescent signals were obtained by a fluorescent microscope with a digital imaging system (Olympus,

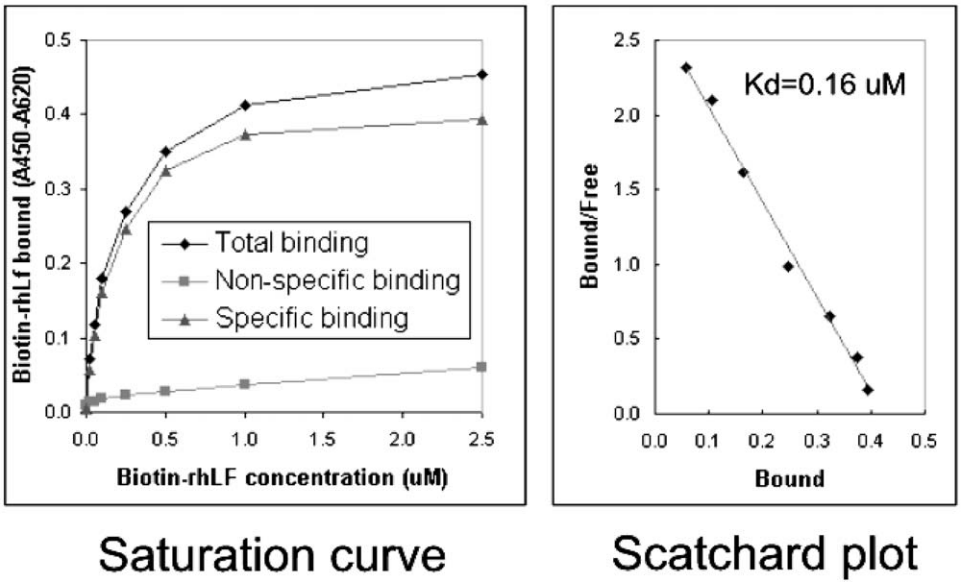

Fig. 2. Specific binding of recombinant human lactoferrin (Lf) to cell membranes from differentiated Caco-2 cells. Several concentrations of biotin-labeled Lf were incubated with Caco-2 cell membranes coating the surface of 96-well ELISA plates in the presence or absence of a 20-fold excess of unlabeled Lf. Total binding was determined by the absorbance at 450 nm minus the absorbance at 620 nm from the wells, to which biotin-labeled Lf alone was added. Non-specific binding was determined by the absorbance at 450 nm minus the absorbance at 620 nm from the wells, to which biotin-labeled Lf was added in the presence of an excess of unlabeled Lf. Specific binding was determined by subtracting the non-specific binding from the total binding. Scatchard plot analysis of the specific binding was performed to estimate the dissociation constant (K_d).

Melville, NY, USA) and a laser scanning confocal microscope (Bio-Rad Laboratories).

Results

We studied the expression of LfR in Caco-2 cells cultured for several different periods. Differentiated Caco-2 cells, which were cultured for 12 days and more, expressed relatively high levels of LfR mRNA and protein, compared to undifferentiated ones, which were cultured for 3 and 5 days (Figure 1). The highest expression level of mRNA was observed on the 19th day of culture. A specific band at a molecular weight of 34 kD was detected under reducing conditions by Western blotting for the LfR. The highest expression level of LfR protein was also at day 19, correlating well with the RT-PCR results. Therefore, we used cells cultured for 19 days for the following studies, i.e., membrane binding assays and cellular internalization studies. The binding assay showed specific binding of Lf to the cellular membrane of differentiated Caco-2 cells with a dissociation constant (K_d) of 0.16 μM (Figure 2). This was close to the K_d of 0.36 μM of Lf binding to recombinant LfR, which was previously reported (Suzuki *et al.* 2001). The post-binding cellular internalization and localization of Lf were studied by using Lf labeled with fluorescent dye. Differentiated Caco-2 cells internalized labeled Lf only from the apical membrane side, but not from the basolateral side (Figure 3a, c). On the other hand, labeled Tf was internalized from only the basolateral side, and not from the apical side (Figure 3b, d). Moreover, internalized Lf was localized to the nuclei (Figure 4a), while internalized Tf was localized to the cytoplasm (Figure 4b). The internalization of labeled Lf was inhibited in the presence of a 100-fold excess of unlabeled Lf, but not unlabeled Tf (data not shown), suggesting that this internalization process is specific for Lf. The internalization of labeled Lf, but not of labeled Tf, was also suppressed in the presence of heparin (Figure 5), indicating that the heparin binding site of Lf is needed for internalization.

Discussion

A specific receptor for Lf has been identified from brush-border membranes of fetal and infant intestines (Kawakami & Lönnerdal 1991), and this LfR has

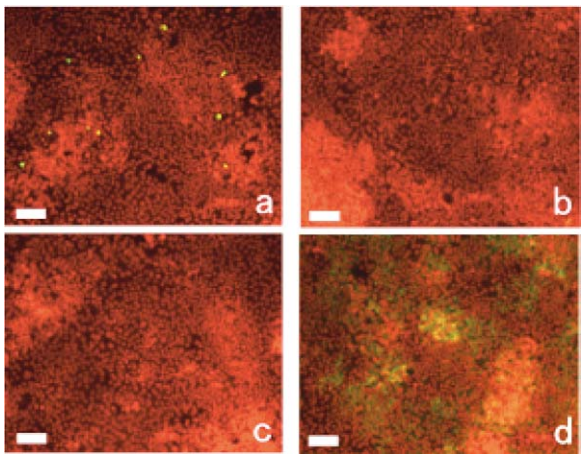

Fig. 3. Cellular internalization of lactoferrin (Lf) and transferrin (Tf) in Caco-2 cells. Caco-2 cells were incubated with Alexa Fluor 488 (green)-labeled lactoferrin (Lf) (a, c) or transferrin (Tf) (b, d) from either the apical (a, b) or the basolateral (c, d) side. The nuclei were stained with TO-PRO-3 (red). Fluorescence images were obtained by fluorescent microscopy. Bar, 100 μm.

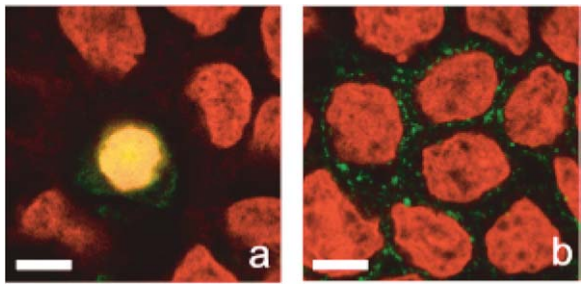

Fig. 4. Intracellular localization of internalized Lf and Tf in Caco-2 cells. Caco-2 cells were incubated with Alexa Fluor 488 (green)-labeled lactoferrin (Lf) from the apical side (a) or Alexa Fluor 488 (green)-labeled transferrin (Tf) from the basolateral side (b). The nuclei were stained with TO-PRO-3 (red). Fluorescence images were obtained by laser scanning confocal microscopy. Yellow color represents co-localization of labeled Lf and nuclei (a). Bar, 10 μm.

been suggested to contribute to the cellular uptake of iron bound to Lf by intestinal epithelial cells (Suzuki *et al.* 2001). First, we demonstrated that differentiated intestinal epithelium-like Caco-2 cells expressed the LfR and that the cellular membrane of the cells bound Lf specifically. Then we histologically examined the post-receptor binding internalization of Lf, comparing it to that of another iron-binding protein, Tf, which has structural homology to Lf and is well-known to be internalized by mammalian cells. We found that Lf was internalized from only the apical side of Caco-2 cells and localized to the nuclei, whereas Tf was internalized from only the basolateral side and localized

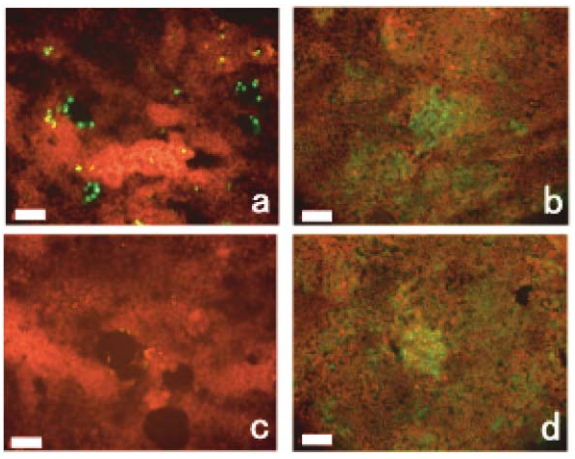

Fig. 5. Effect of heparin on the cellular internalization of Lf and Tf in Caco-2 cells. Caco-2 cells were incubated with Alexa Fluor 488 (green)-labeled lactoferrin (Lf) from the apical side (a, c) or Alexa Fluor 488 (green)-labeled transferrin (Tf) from the basolateral side (b, d) in the presence (a, b) or absence (c, d) of heparin. Nuclei were stained with TO-PRO-3 (red). Bar, 100 μm.

to the cytoplasm. These findings suggest that Lf and Tf have different systems of cellular internalization and also have different physiological roles in the epithelial cell.

Cellular internalization of Tf was previously observed in Caco-2 cells (Hughson & Hopkins 1990). Internalization of Tf from the basolateral side is thought to be involved in iron uptake from the extracellular body fluid. The internalization of Lf from the apical side of Caco-2 suggests that Lf internalization may be involved in the iron uptake from the luminal fluid. On the other hand, the nuclear localization of Lf may propose another function of Lf in the nuclei of those cells. It has been demonstrated that Lf binds to a specific DNA sequence (He & Furmanski 1995) and activates the transcription of a specific gene (Son *et al.* 2002).

Nuclear localization of Lf has been reported in human erythroleukemic cells (Garré *et al.* 1992). Recently, the N-terminal portion of Lf, which is rich in basic amino acids, has been identified as a signal peptide, which is responsible for the ability of the cellular internalization and the nuclear localization of Lf in several epithelial cell lines, i.e., HeLa, U87MG and 5637 (Penco *et al.* 2001). This portion is also recognized as a heparin-binding site of Lf (van Berkel *et al.* 1997). We demonstrated that heparin inhibited the cellular internalization of Lf in this study. This result suggested that the N-terminal portion should be important for the internalization of Lf in Caco-2 cells,

which is in accordance with the previous report for other epithelial cells.

Systemic effects of oral administration of Lf on immune function have been reported in humans and animals. However, the mechanisms behind the action of orally administrated Lf have not been understood. In this study, we demonstrated that Lf was internalized by intestinal epithelium-like Caco-2 cells from the apical side and localized to the nuclei, which suggests that ingested Lf might modulate the function of intestinal epithelium through gene regulation. This may explain in part the mechanism behind the systemic effects of orally administered Lf.

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